6

The Development, Characterization and Application of Water Soluble Chitosan

Zanariah Ujang[1], Mazita Diah[1],
Ahmad Hazri Abdul Rashid[1] and Ahmad Sukari Halim[2]
[1]*SIRIM Berhad,*
[2]*Universiti Sains Malaysia,*
Malaysia

1. Introduction

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is produced commercially by the deacetylation of chitin, a long-chain polymer of N-acetylglucosamine which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. The degree of deacetylation (%DD) can be determined by NMR spectroscopy, and the %DD in commercial chitosans is in the range 60-100 %. A common method for the synthesis of chitosan is the deacetylation of chitin using excess concentrated sodium hydroxide as a reagent. It has the same β- (1-4)-D-glucopyranose unit's backbone as cellulose, except that the 2-hydroxy is replace by an acetamide group. Owing to its specific structure and property, chitosan has attracted significant interest in a broad range of areas such as pharmaceutical (Kato et al., 2003, Kumar et al., 2004), biomedical (Suh and Matthew, 2000, Tucci and Rigotti, 2003, Ng and Swami, 2005) water treatment(Northcott et al., 2005, Crini, 2005), cosmetics (Rinoudo, 2006, Sun et al., 2006), agriculture (Boonletniruni et al., 2008,El Hadrami et al., 2010) and food industry (Ham-Pichavant 2005,De Lima et al., 2010). As chitosan is a linear cationic biopolymer, it is only soluble in acidic aqueous solution in which the primary amino groups are protonated and precipitates when neutralized. The presence of rigid crystalline domains, formed by intra-and/or intermolecular hydrogen bonding, is considered to be responsible for the poor solubility of chitosan in high pH solutions (Nishimura et al., 1991).) The extended applications of chitosan, is therefore frequently limited by its solubility behaviour. The solubility of chitosan can be improved by depolymerization and its chemical modification (Cravotto et al., 2005). Chitosan has reactive amino, primary hydroxyl and secondary hydroxyl groups which can be used for chemical modifications under mild reaction conditions to alter its properties.

In particular, chitosan and its derivatives have been considered as biomaterials because of their biocompatibility, biodegradability, low immunogenicity and biological activities. (Hirano 1999, Molinaro et al., 2002) Chitosan has been well known to possess valuable properties for biomedical applications [Li et al, 1997] and being able to accelerate the healing of wound (Kwaeon et al., 2003, Khnor and Lim, 2003). It has also been documented that

chitosan confers considerable antibacterial activity against a broad spectrum of bacteria [Liu et al., 2001, Zhao et al., 2003]. Owing to the advantages, some of the applications of chitosan have included wound dressing, gauzes and medical sutures (Lee et al., 2000, Mi et al., 2001).

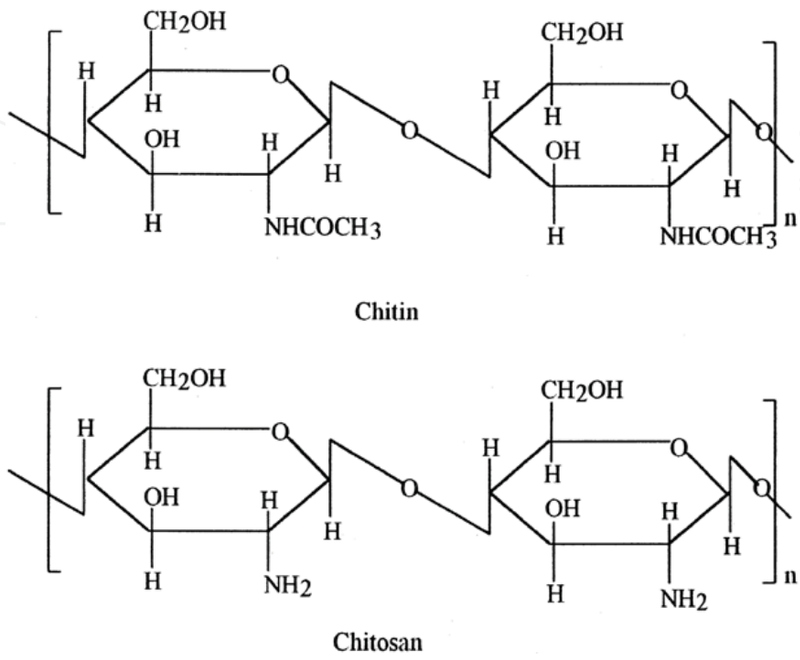


Fig. 1. The structure of chitin and chitosan

In this chapter we present the development of water soluble N,O-carboxymethylchitosan (NOCMC) and chitosan hydrolysates from an enzymatic process and their application in wound healing. Characterization study such as FTIR, DSC and TGA were performed on both materials. In addition, their biological properties such as anti-oxidant effect, inhibition of melanin synthesis and cytotoxicity effect were carried out in *in-vitro* studies. *In-vivo* biocompatibility of these materials and their efficacy as wound dressing materials were tested using animal models.

2. Production of water soluble chitosan

2.1 Preparation of N,O-carboxymethyl chitosan (NOCMC) and characterization

The method used is the carboxymethylation of chitosan through direct alkylation reaction using monochloroacetic acid as the reagent. Due to similarities in structure, this method follows closely the method of carboxymethylation of cellulose to form CM-cellulose. Chitosan was initially swelled with isopropanol followed by the formation of a Na-chitosan complex on reaction with NaOH. Derivatisation was achieved using chloroacetic acid (CAA). The chitosan derivative was washed and the pH of the solution was adjusted to the required pH.

Freshly prepared NOCMC was freeze dried into powder form and stored in the refrigerator prior to further use. NOCMC concentrations of up to 10% solution in water were prepared for *invitro* analysis. 1% and 5 % solution were prepared and cast into thin films for used in *invivo* studies.

2.2 Characterization

2.2.1 FTIR and NMR analysis

Fourier Transform Infra Red (FTIR) was carried out using instrument model Perkin Elmer Spectrum 2000. The spectra were recorded at 48 scanning at resolution of 4 cm^{-1}.

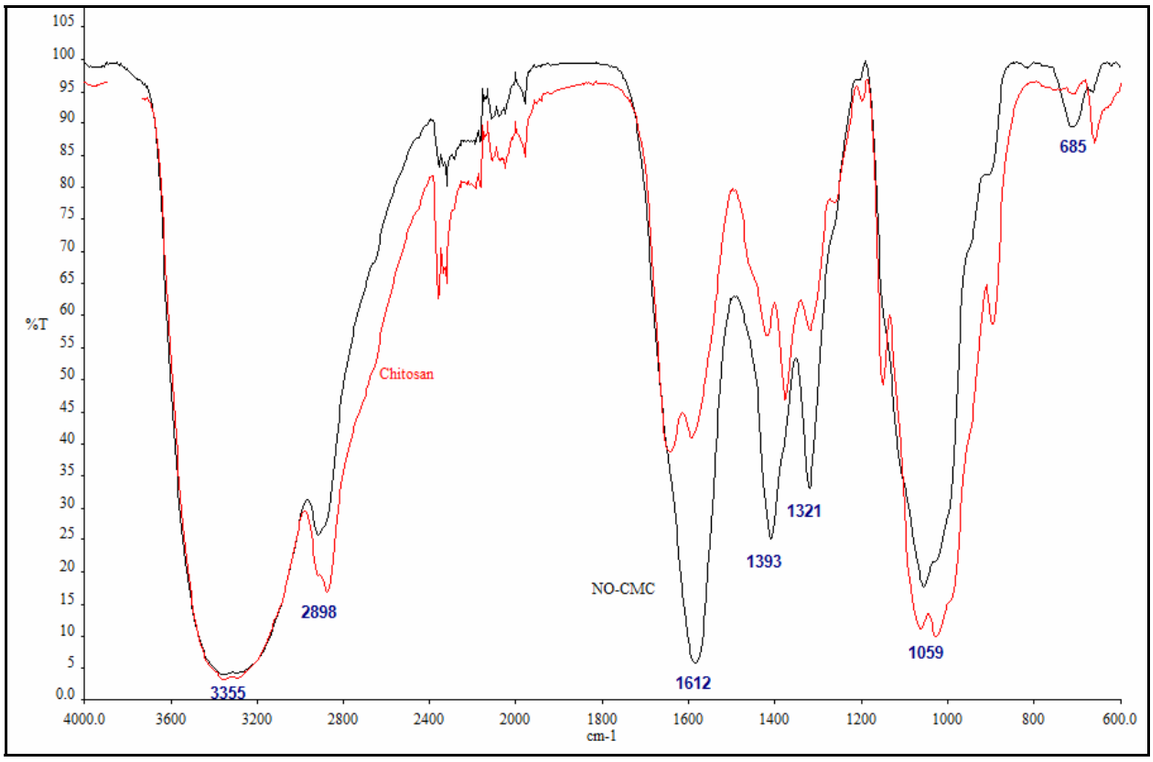


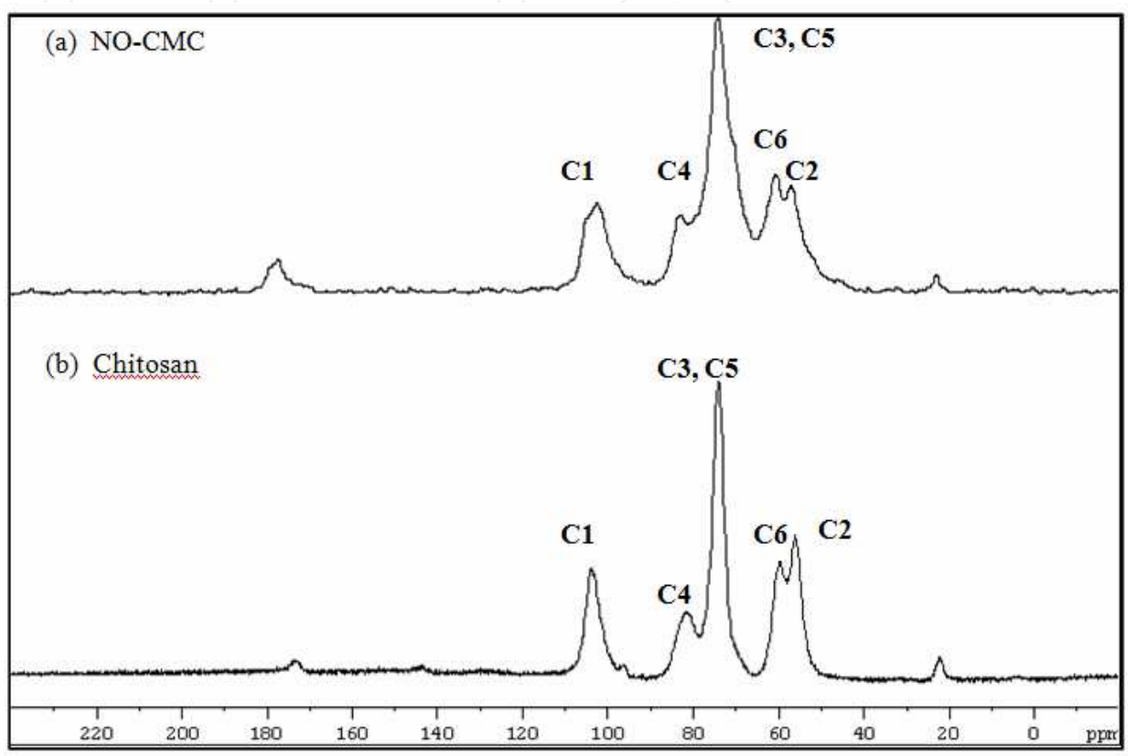
Fig. 2. FTIR spectrum of chitosan and NO-carboxymethyl chitosan

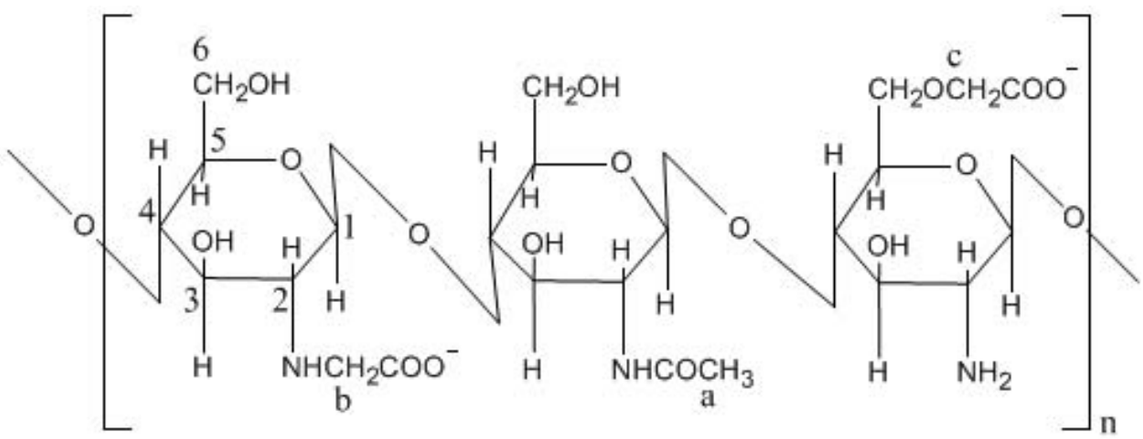
Figure 2 shows the FTIR spectrum for chitosan (bottom, red) and N,O-carboxymethylchitosan (top, black). The characteristic bands for chitosan was observed in the infrared spectrum around 3294 cm^{-1} and 2874 cm^{-1} attributed to N-H, O-H and C-H stretching bond. These bands are also present for N,O-carboxymethylchitosan spectrum. The characteristic peaks at 1647cm^{-1} and 1376 cm^{-1} were assigned as amide I and amide III band of chitosan, respectively. The band at 1150 cm^{-1} was the asymmetric stretching of C-O-C bridge. Bands at 1062 cm^{-1} and 1027 cm^{-1} were assigned to the skeletal vibration of C-O stretching (Brugnerotto *et. al*., 2001).

The carboxymethylation provoked structural changes which were clearly identified by comparing the infrared spectra of chitosan and N,O-carboxymethylchitosan. Occurrence of intrinsic peaks at 1583 cm^{-1} and moderate band at 1409 cm^{-1} in N,O-carboxymethylchitosan spectrum, which were attributed to the symmetric and asymmetric axial deformation of COO, respectively, confirmed the introduction of the carboxymethyl group (Zhao et. al, 2002, Mourya et. al., 2010). Band corresponding to the polysaccharides skeleton, including the vibration of the glycoside bonds, C-O and C-O-C stretch in range 1062-1027 cm^{-1} confirmed that the back bone of the derivative is still intact.

^{13}C Magic Angle Spin Nuclear Magnetic Resonance (MAS NMR) spectrum was obtained using 400 MHz Brucker model. ^{13}C MAS NMR in the solid state provided information on the molecular skeleton of chitosan and its derivative. The NMR spectrum (Figure 3),

correspond to the structure of chitosan and have defined as C1 peak (δ103.7), C2 (δ56.1), C3 (δ74.1), C4 (δ81.5), C5 (δ74.1) and C6 (δ59.6). C3 and C5 peaks appear as an only peak at δ 74.1 ppm (Paulino, 2006). In N,O-carboxymethyl chitosan, the basic structure of chitosan is still present but a new peak was observed at δ 177.7 ppm. This peak corresponds to the carbon from carboxyl group (COOH), due to the incorporation of carboxylmethyl group in the chitosan structure.





(c)

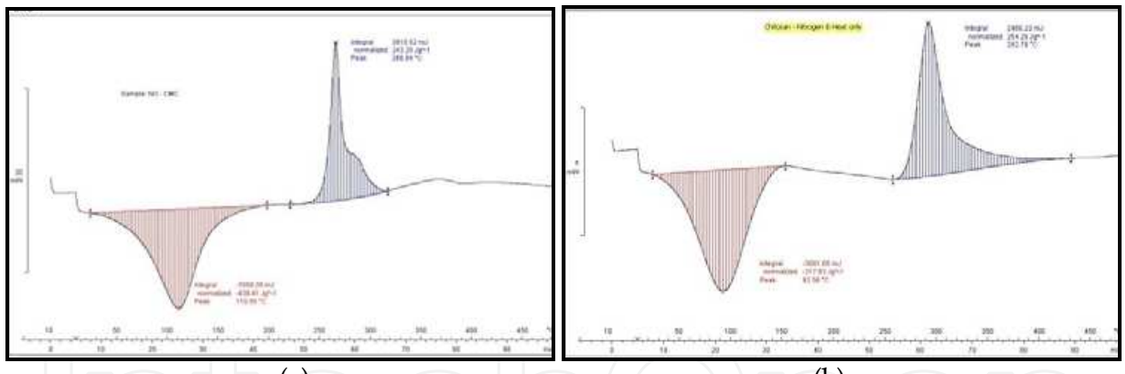
Fig. 3. ^{13}C MAS NMR spectra of (a) N,O-carboxymethyl chitosan and (b) chitosan. (c) structure of carboxymethylchitosan

2.2.2 Thermal analysis

Differential scanning calorimeter (DSC) measurements were performed in a DSC-910 thermal analyzer from TA Instruments, USA. The DSC curves were performed under dry nitrogen atmosphere (50-100mL min^{-1}) using different sample mass as 2-5mg and heating rate 5°C. Accurately weighted samples (~0.1mg) were placed into a covered aluminum sample holder with a central pin hole. Indium metal (99.99%) was used to calibrate the DSC modulus in relation to temperature and enthalpy. An empty sample holder was used as reference and the runs were performed by heating the samples from 25°C up to 110°C followed by an isothermal at 110°C for 15min. The samples were reweighted and heated from 10°C up to 480°C.

Thermogravimetric analysis (TGA) were performed in a Perkin-Elmer Model TGA-7. TGA was carried out to study the thermal degradation of chitosan and NO-CMC. The samples were heated at 10°C/min from 30°C to 600°C under nitrogen atmosphere. At 600°C the nitrogen gas was replaced with oxygen while maintaining the heating rate of 10°C from 600°C to 800°C.

As polymer processing frequently involves its melting and extrusion, the studies on its thermal properties and stability are very important to support the technological applications of the polymer. Chitosan as well as their derivatives have good film forming properties and they can be processed as membranes, solutions, gels, pastes and nanoparticles. Some studies on the thermal degradation of chitosan (Holme et al., 2001) and carboxymethylchitosan (Kittur et al., 2002) show that the introduction of substituents on the amino groups of chitosan decreases its thermal stability.



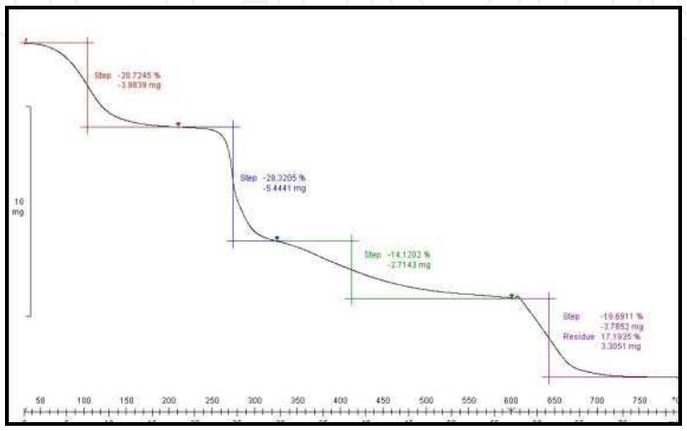
(a) (b)

Fig. 4. DSC thermogram of (a) chitosan and (b) N,O-carboxymethylchitosan

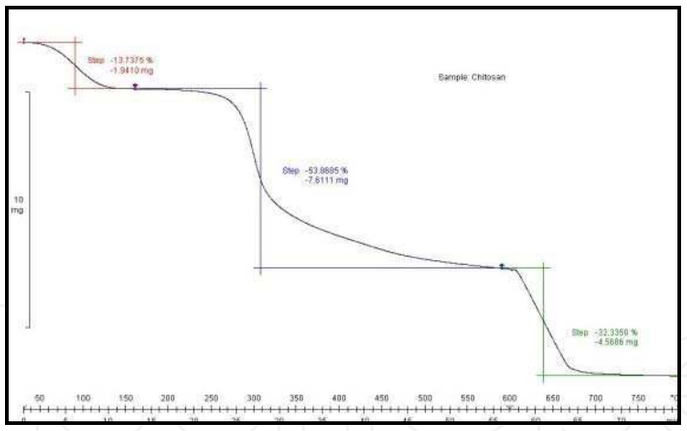
The DSC thermograms of chitosan and NOCMC (Figure 4 a & b) were characterized by two thermal events: the first endothermic and the second exothermic. The endothermic event appeared at 92.56°C for chitosan and 110.55°C for NOCMC corresponding to water evaporation. The exothermic event appeared at 292.76°C for chitosan and 266.84°C for NOCMC corresponding to the decomposition of the polymers. This decomposition event is concluded by the failure of the polymer to show any readable thermograms on cooling and reheating. A glass transition temperature (T_g) was also not observed in thermogram of chitosan and NOCMC indicating its amorphous characteristics.

Polysaccharides have a strong affinity for water, and in the solid state these macromolecules may have disordered structures which can be easily hydrated (Galo & Patricia 2004). Figure 5 shows the TGA chromatogram for chitosan and NOCMC. The TGA of chitosan exhibit a

small curve at 90°C while NOCMC exhibit a small curve at 100°C mainly from adsorbed humidity. NOCMC loses substantially more water (20.72%) compared to chitosan (13.73%) which can be attributed to the higher hydrophilicity of NOCMC leading to higher bound water as compared to chitosan. According to Mourya et al., The increase in water holding capacity with the increasing N-deacetylation and carboxymethylation is attributed to newly created hydrophilic centers (amine and carboxymethyl) in the polymer chain (Mourya et.al., 2010). Figure 5A shows the decomposition peaks at 310°C and 53.87% corresponding to decomposition of glucosamine group. Figure 5B showed decomposition peaks at 270 °C and 410°C corresponding to decomposition of carboxylic group and glucosamine unit.



(a)



(b)

Fig. 5. TGA thermogram of (a) chitosan and (b)N,O-carboxymethylchitosan

2.3 Preparation of oligo chitosan

Oligochitosan samples were prepared by an enzymatic hydrolysis reaction. A number of cellulose hydrolytic enzymes such as chitosanase (Shee. et. al., 2008), chitinase (Lopatin, et.al., 1995), and cellulase (Xie, et. al., 2009) may be utilized for the hydrolysis, with the two most efficient being chitosanase and chitinase with chitosan being their natural substrate. In this work pure chitosanase from *Streptomyces species,* supplied by Sigma Chemical Co, St Louise USA, was chosen as the hydrolytic enzyme. The enzyme loading was 1U/30 g

chitosan/L solution. The hydrolysis reaction was run 50°C and reaction without enzyme was also run for control.

The enzymatic hydrolysis was performed in 10 litres of 3% acidic chitosan solution. Hydrolysed samples were taken for 0, 1,2, 4,6,7 and after 24 hours and the enzyme was inactivated at 100°C. Viscosity for all samples were measured upon cooling using a Brookfield Viscometer model DV -11+ Pro with spindle 29 at 100rpm.

All the oligo chitosan samples were freeze dried and refrigerated prior to further use to prevent further autohydrolysis. Oligo chitosan samples with concentrations of up to 5% solution in water were prepared for *invitro* analysis. 1% and 5 % solution were prepared and cast into thin films for used in *invivo* studies.

2.4 Characterization

2.4.1 Measurement of viscosity

Degradation of chitosan may be achieved by chemical (acid) hydrolysis (Qin et al 2002, Jia and Shen 2002, Hsu et al. 2002) or enzymatic hydrolysis (Qin et al., 2003, Shin- ya et al., 2001) Although hydolysis of chitosan with acids is fast and less expensive, the chemical structure of residues is easily damaged. The use of enzyme for degradation is generally more suitable as the course of the hydrolysis process and the product distribution are easier to control. Moreover, enzymatic hydrolysis may lend itself to production of less polymerized chitosan products which retain their original polymeric nature.

In our reaction, the enzymatic hydrolysis reaction was performed up to 7 hours with the final oligochitosan solution having a viscosity of around 400 centipoise down from the initial viscosity of 3% chitosan at 5400 centipoise (Figure 6).

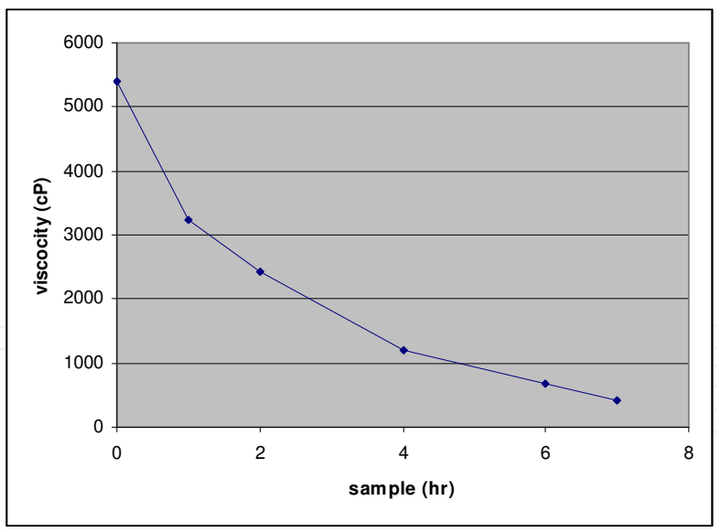


Fig. 6. Measurement of viscosity of 3 % chitosan solution after addition of enzyme

2.4.2 FTIR analysis

Figure 7 shows the FTIR spectrum for oligochitosan samples taken at several time course. Its structural composition were found to be similar to the original structure of chitosan, except for the peak occurring at 1550 cm^{-1}. This peak at 1550 cm^{-1} is assigned to the COO- residue shifting from the acetic acid to amine residue of chitosan (Seung et al., 2001).

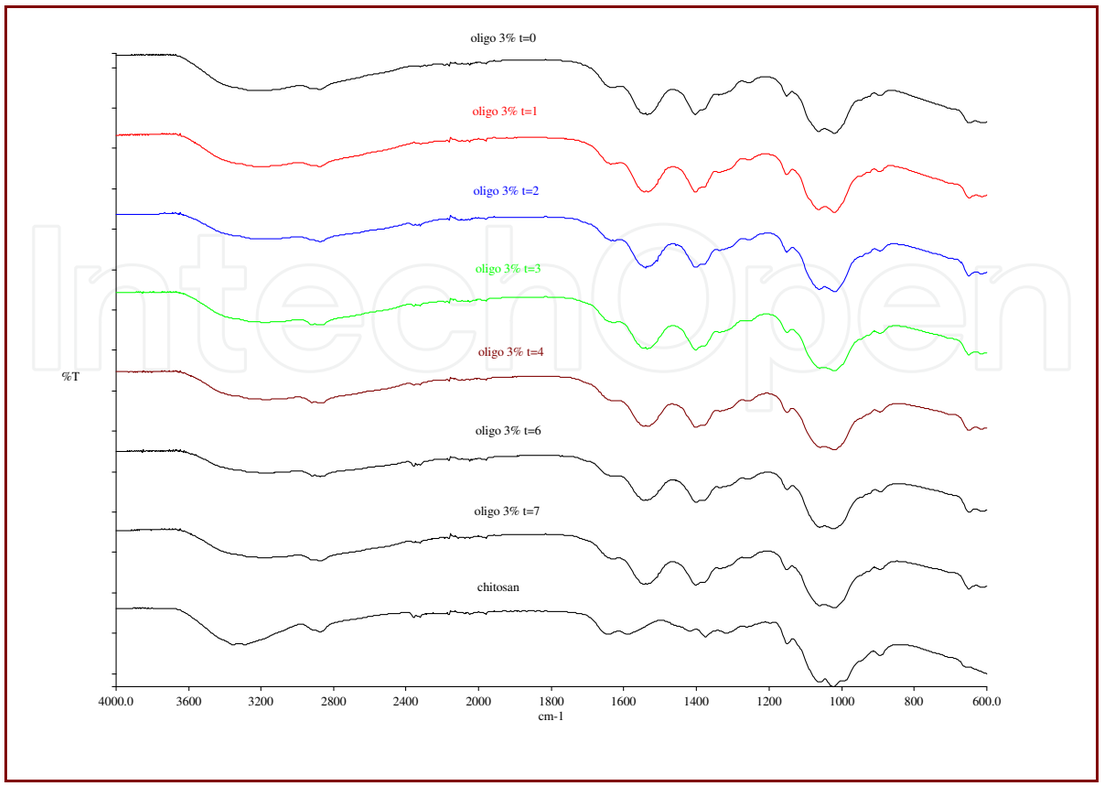


Fig. 7. FTIR spectrum for chitosan and oligochitosan

2.4.3 Thermal stability study

Sample	Water evaporation onset temperature (°C)	Heat capacity (mJ)	Decomposition temperature (°C)	Heat capacity (mJ)
Chitosan	92.56	3081.00	292.79	2466.22
3% Oligo chitosan t = 1 hr	88.87	1128.74	284.64	620.87
3% Oligo chitosan t = 3 hr	79.36	667.31	281.98	1441.15
3% Oligo chitosan t = 7 hr	84.36	751.94	281.68	743.81

Table 1. Summary of DSC thermograms

The DSC thermograms of chitosan and oligochitosan (Figure 8) were characterized by two thermal events: first endothermic and the second is exothermic. The endothermic event appeared at 92.56, 88.87, 79.36 and 84.36°C, for chitosan, oligochitosan t =1 hr, oligochitosan t =3 hr and oligochitosan t =7 hr respectively corresponding to the onset of the evaporation of bound water, the event lasting until slightly over 100°C,. The exothermic event appeared at 292.76, 284.64, 281.98 and 281.68°C for chitosan, oligochitosan t =1 hr, oligochitosan t =3 hr and oligochitosan t =7 hr respectively, corresponding to the decomposition of the polymers as explained in a previous work (Mourya et al. 2010). The degradation temperature of chitosan is

higher compared to oligochitosan due to the higher molecular weight chitosan and the higher number of bonds that need to be broken. Reduction in molecular weights is also translated to viscosity reductions from chitosan to oligochitosan (Figure 6).

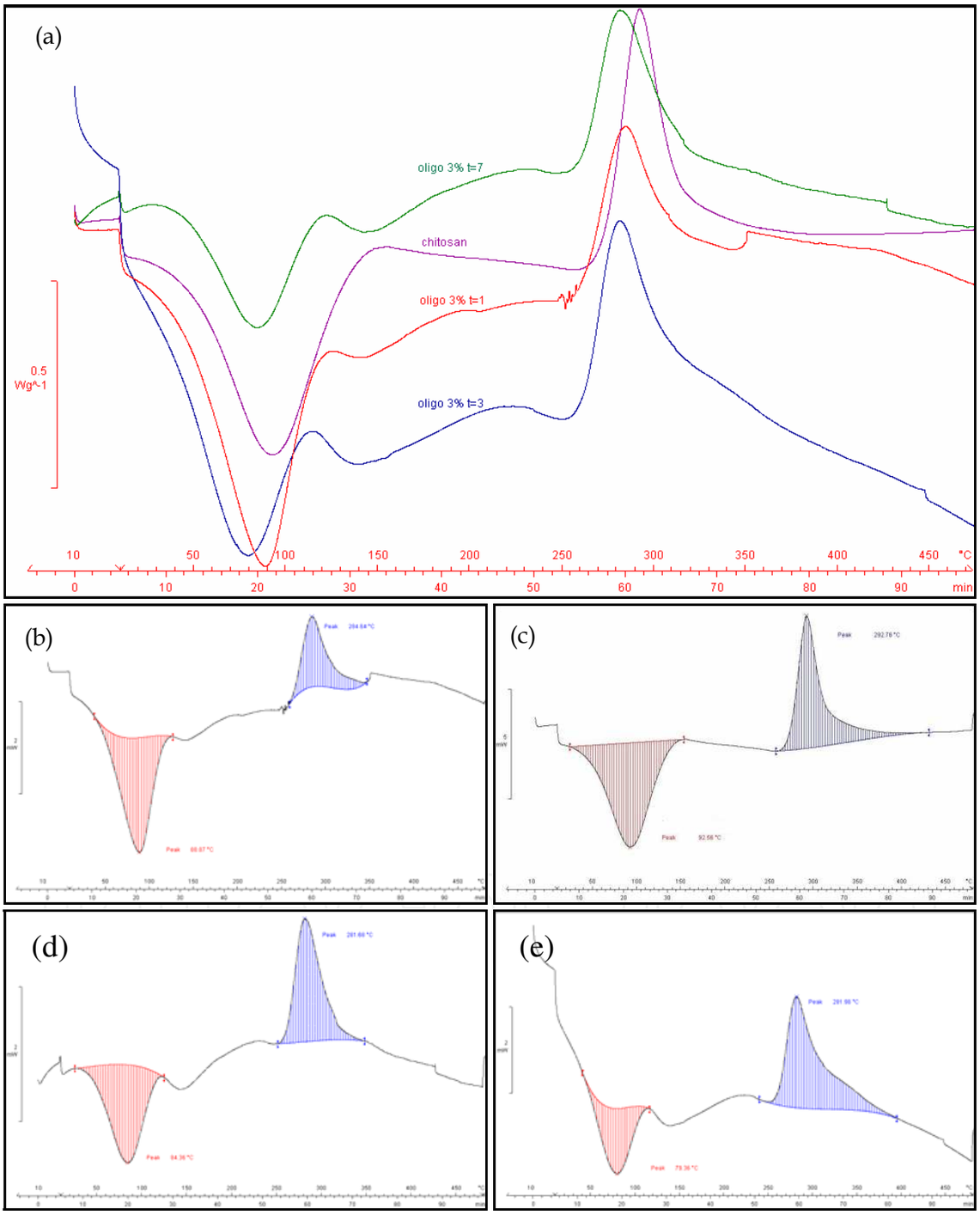


Fig. 8. DSC thermogram of (a) overlay (b) chitosan (c) oligochitosan t = 1 hr (d) oligochitosan t = 3 hr and (e) oligochitosan t = 7 hr

3. Biological activity

The main research associated with this work was to investigate the biological activities of the water soluble chitosan in the area of wound healing in general. This involved the assaying of the chitosan samples for inhibition of melanogenesis, anti-oxidant and wound healing which forms part of a series of bioactivities in wound healing technology.
Apart from the biological activity of interest it is also important to ascertain the toxicity limits of the chitosan samples with respect to its contact with the cells and tissues of the human body. Two different toxicity assays were performed namely the MTT cell viability which is an assessment on the raw materials and was carried out concurrently with the melanogenesis assay on the same cells. The other toxicity evaluation is the direct contact method whereby the final wound healing product is assessed when in contact with the skin cells.

3.1 Melanogenesis assay

Melanogenesis is the process of the production of melanin by certain melanin producing cells such as melanocytes which forms part of the skin epidermis layer. The melanogenesis assay is the determination of the ability of the samples to inhibit the production of melanin by selected cells. A high melanin inhibition activity also imparts on the materials potential prospect to be utilized as functional skin care product in the treatment of over expression of melanin such as hyperpigmentation.

3.1.1 Materials

The B16-F1 (CRL-6323) melanoma cells were purchased from American Type Culture Collection. Cells were first cultured in DMEM medium supplemented with antibiotics (100U/mL of penicillin and 100U/mL of streptomycin) and 10% heat-inactivated fetal calf serum (Gibro-BRL) and maintained at 37°C in a humidified incubator containing 5% CO_2 . Melanin synthetic, kojic acid, diphenyl tetrazolium bromide (MTT) and trichloroacetic acid (TCA) were purchased from Sigma.

3.1.2 Method

The B16-F1 melanoma cells were seeded into 24 well plates at $1X10^5$ cells/ml and cultured until they reached 90% confluence. They were then treated with sample at various sample for 24 hrs using kojic acid as standard. Cells were then harvested for melanin content and MTT assay.

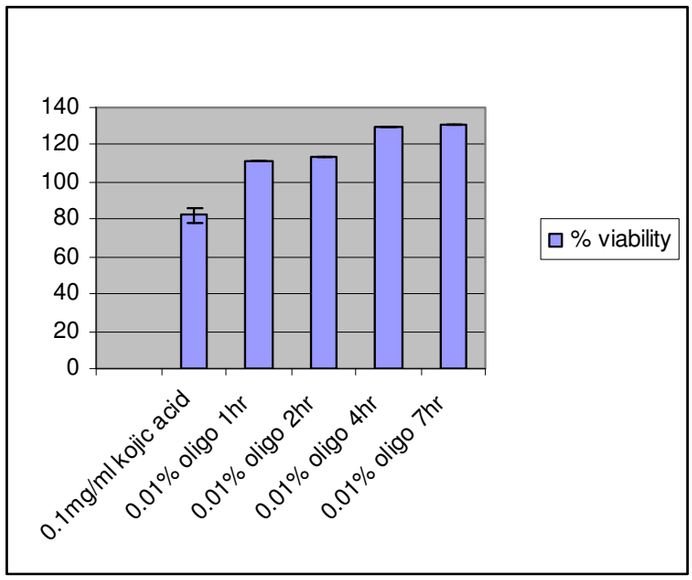
3.1.3 Melanin content

The extracellular melanin content of cells treated with and without extracts were determined. Medium cultured were collected and incubated with 5% TCA at room temperature. Medium were centrifuged then dissolved in 1 N NaOH and shake for 15 minutes at room temperature. Absorbance was measured using Spectrophotometer at 475 nm and compared against the standard curve of melanin synthetic (Sigma).

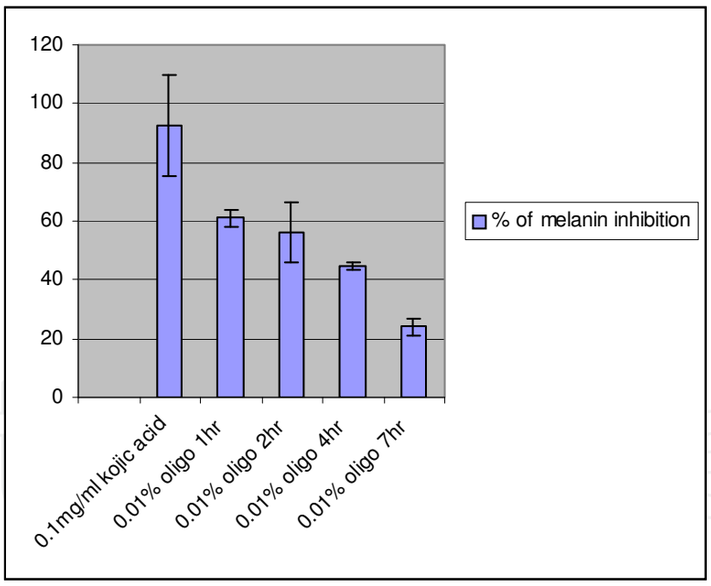
3.1.4 Cell viability (MTT)

5mg/ml MTT agent was added into each well and cells incubated for 3 hours at 37°C in a humidified incubator containing 5% CO_2. Cells were then centrifuged and dissolved with DMSO**.** Absorbance was measured using Spectrophotometer at 530/690 nm.

3.2 Inhibition of melanin formation by water-soluble chitosan
3.2.1 Inhibition of melanin formation by oligochitosan



(a)



(b)

Fig. 9. Effects of oligochitosan on melanin production and cell viability in B16-F1 melanoma cells. Cells were treated with sample for 48 hrs. (a) Cell viability measured by MTT assay on extracellular melanin content. (b) Effect of oligochitosan on extracellular melanin content measured by MTT assay.

Melanin formation is the most important determinant of mammalian skin color (Hearing, 2005). In this study, melanin assay was performed on the chitosan samples to determine its depigmentation effect. MTT assay was performed to determine the toxicity of chitosan on

melanocytes. Measurements of extracellular melanin content in the melanocytes are shown in figure 9b, while % cell viability is shown in figure 9a. Kojic acid was used as standard which gave the % melanin inhibition of 92.31 ± 17.2 with % cell viability 82.34 ± 4.02. The oligochitosan samples inhibit the formation of melanin in skin cells with 60% inhibition showed by the hydrolysis sample after I hour. All samples tested are non-toxic to the skin cells. The ability of the oligo chitosan to regulate melanin formation is useful in reducing scar color during wound healing and recovery process.

3.2.2 Inhibition of melanin formation by N,O-carboxymethylchitosan

No melanin inhibition effect was observed with the NOCMC samples. The samples are non toxic to melanocytes with cell viability of more than 100% observed in all samples.

3.3 Anti- oxidant effect

ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay is basically a radical cation decolourisation test, which is also a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. Any substance which possesses anti-oxidant activity has a high potential of being a good wound healing material.

3.3.1 ABTS method

Phosphate buffer (PBS) 5mM, pH 7.4

The Solution A was prepared with 0.13609g of KH_2PO_4 (acidic) was dissolved in 200 ml of distilled water. The solution B was prepared with 2.1742 of K_2HPO_4 (alkaline) were dissolved in 200 ml of distilled water. Solution B was mixed with solution A using a magnetic stirrer along with pH meter to control the pH at 7.4.

ABTS Stock Reagents

ABTS stock reagents were prepared by dissolving 0.0768 $ABTS^{\cdot+}$ and 0.0132g potassium persulfate in 20ml of distilled water. The stock reagent was kept in dark for 12 to 16 hours at room temperature before use. This stock reagent can be kept in the freezer under 20°C until further use.

ABTS working solutions

Working solution of ABTS was prepared by adding 19 ml of phosphate buffer (PBS) 5mM, pH 7.4 to 1 ml of the stock reagent.

Method for ABTS

A total of 200µl of the working solution was added to 20µl of the plant extracts to make a final volume of 220 µl and the absorbance was read after 6 minutes incubation. The absorbance value was measured at 690nm. Ethanol/ distilled water were used as the negative control with the same volume as the extracts. L-ascorbic acid (20 µl) plus ABTS reagent (200 µl) was used as positive control. This assay was performed in triplicates.

$$\% \text{ Inhibition} = \frac{\text{A neg control-A simple}}{\text{A neg control}} \times 100\%$$

L- ascorbic acid was used as standard in this assay and are prepared fresh prior to use.

3.3.2 Anti-oxidant activity

The anti-oxidant activity of chitosan and its derivatives has indicated that the active hydroxyl and amino groups in the polymer chains may take part in free radical scavenging and contributed to the anti-oxidant activity. The contents of active hydroxyl, amino, amido groups in their polymer chains as well as molecular weight affect the anti-oxidant activity of chitosan and derivatives (Feng et al., 2008, Guo et al., 2005)

Sample	IC_{50} (mg/ml)
Standard - Vit.C	0.09 ± 0.00
OC (1 hr)	3.99 ± 0.13
OC (2 hrs)	3.47 ± 0.048
OC (3 hrs)	4.31 ± 0.10
OC (6 hrs)	3.81 ± 0.05
OC (7 hrs)	4.66 ± 0.20
NOCMC	0.98 ± 0.07

Table 2. IC_50 value of Oligo chitosan and NO carboxymethyl chitosan

Table 2 shows the concentration of oligo chitosan and NOCMC in inhibiting 50 % of ABTS radical formation. Oligochitosan displayed IC_{50} values of between 3.5 to 4.6 mg/ml while NOCMC is more effective as a free radical scavenger compared to oligo chitosan. Sun et al, 2007 has shown that carboxymethyl chitosans showed molecular weight dependent scavenging activity against superoxide anion.

3.4 Cytotoxicity test-direct contact method

The direct contact cell culture evaluation of materials was employed to assess the cytotoxicity of the biomaterials. Fibroblast ($6x10^4$ cells/ml) in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) was seeded in each well of 24-well culture plates (Nunc, Denmark). Plates were incubated overnight at 37°C in humidified 5% CO_2 atmosphere until a monolayer (> 80% confluency) was formed. The culture medium was aspirated and test material pieces (chitosan film, 0.5 cm^2) were placed carefully on the cell layer in a minimum culture medium. In order to evaluate the effect of chitosan film on fibroblast cells, cultures were incubated with or without test materials for 24, 48 and 72 hours. Morphology of cells was assessed using inverted microscope and quantitative evaluation of cytotoxicity was done using tetrazolium salt (MTT) reduction assay.

3.4.1 Cytotoxicity of the water soluble chitosan

Oligo Chitosan film was found to be non-toxic to human skin fibroblasts. Our results show that oligo chitosan film prepared from 1% solution did not induce any cytotoxicity effect to fibroblasts in vitro as they represent more than 80% of cells viability . Figure 10 shows the cytotoxicity results for oligochitosan on fibroblasts culture using direct contact test. Results demonstrated that, O-C 1% derivatives exhibit high percentage of cell viability in terms of their biocompatibility. Time exposure did not significantly affect the number of fibroblasts growth.

Test Materials	% of Cell Viability ± SD		
	24 HOURS	48 HOURS	72 HOURS
O-C 1% (1H)	89.92 ± 15.15	99.05 ±18.6	99.23 ± 4.40
O-C 1% (3H)	92.52 ± 16.26	102.15 ± 23.01	99.35 ± 5.88
O-C 1% (6H)	94.19 ± 14.83	103.22 ± 15.53	98.50 ± 6.13

Table 3. Cell viability of oligo chitosan tested at 24, 48 and 72 hours. Results represent the mean viability of six replicates on fibroblast obtained from different donor.

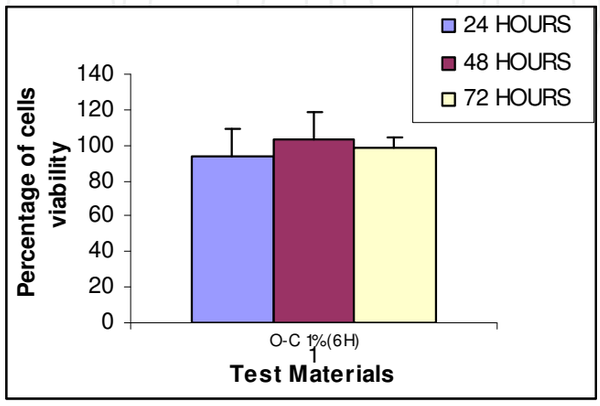


Fig. 10. Quantitative cytotoxicity test: Fibroblasts survival in the presence of oligo chitosan sample(direct contact) at 6 h. MTT reduction capacity values are expressed as the percentage of control values obtained by cell incubations in the absence of test materials. Graph represents the mean value of fibroblast cell viability from different donor.

Test Materials	% of Cell Viability ± SD		
	24 HOURS	48 HOURS	72 HOURS
NOCMC 1%	75.37 ± 18.47	58.82 ± 5.14	44.17 ± 14.53

Table 4. Cell viability of NOCMC tested at 24, 48 and 72 hours. Results represent the mean viability of three replicates on fibroblast obtained from different donor

NOCMC showed a higher toxicity effect to fibroblast compared to oligochitosan especially at longer incubation period as cell viability drop to less than 50%.

Chitosan is a cationic polymer having an amino group in its chemical structure. The *N*-acetylglucosamine moiety in chitosan is structurally similar to glycosaminoglycans (GAGs), heparin, chondroitin sulphate and hyaluronic acid in which they are biocompatible, and have specific interactions with various growth factors, receptors and adhesion proteins besides being the biologically important mucopolysaccharides in all mammals. Therefore, the analogous structure in chitosan may also exert similar bioactivity and biocompatibility. In our previous research paper, *in vitro* biocompatibility evaluation of biomedical-grade oligochitosan) and *N,O*-carboxymethyl-chitosan (NOCMC) derivatives (oligochitosan 1%, oligochitosan 5%, NO-CMC 1% and NO-CMC 5%) correlated well with *in vivo* results (Lim *et al*., 2007), in which oligochitosan 1% remained the most cytocompatible chitosan film compared with oligochitosan 5% and NO-CMC. This suggests the reliability of the *in vitro* model as a tool to evaluate the cytotoxicity of biomedical grade chitosan.

4. *In vivo* study

In-vitro studies have demonstrated the excellent prospect for the water soluble chitosan to be used as wound healing materials. However this study need to be extended to an in vivo study on an animal model (rats and rabbits) to further elucidate its effectiveness for this particular application. The study was undertaken in two parts namely:

- the biocompatibility study on rats via the implantation method to determine the reaction of the animal to foreign material implanted into their bodies,
- the wound dressing study on rabbits to determine the effectiveness of the water soluble chitosan as in the treatment of surface wounds.

Both methods are described below.

4.1 Implantation method

4.1.1 Creation of subcutaneous pockets

Healthy male Sprague-Dawley rats weighing between 300-500g were used in this study. The rats were subjected to the implantation period of 4 days, 7 days, 14 days, 21 days and 28 days. On the day of implantation, the rat was placed in ventral position and immobilized on their abdomen for the surgery. The dorsum of the rat was shaved. Immediately before operation the rat was anaesthetized with intramuscular injection of Ketamine 35.0 mg/kg and Xylazine 5.0 mg/kg on gluteal area. When fully anesthetized, the shaved area was cleaned three times with povidone iodine. The operation site was then isolated with sterile towel. Wounds were created under sterile technique for subcutaneous implantation. Paravertebrally, at both side of the spinal column, two longitudinal incisions were made through the full-thickness incisions of the skin. Subsequently, lateral to the incisions a subcutaneous pocket was created by blunt dissection with scissors. The implant was inserted in these pockets. The contra lateral pocket served as control site. Finally the wounds were carefully closed intracutaneously with ethilon 3-0.

At 4 days post-implantation, the rats were euthanized and the implant and the control with surrounding tissue were retrieved. The remaining implants and control site were retrieved at 7 days, 14 days, 21 days and 28 days post-implantations. Implants from all rats were randomly chosen, sectioned and analyzed by histological examination.

4.1.2 Microscopic examination

Immediately after retrieval, specimens were fixed in a 10% buffered formalin solution. Subsequently, the tissue blocks were dehydrated in a series of ethanol (from 10% (v/v) to 100%) and embedded in paraffin block. After polymerization, non-decalcified thin (10 μm) sections were prepared in a transversal direction to the axis of the implant using a modified sawing microtome technique. Six histological sections were made for each tissue block. These sections were stained with Hematoxylin and Eosin (H&E) staining and examined using a light microscope. A light microscope was used for the histological evaluation. The histological evaluation consisted of a concise description of the observed specimens and an analysis of the tissue response. The histological evaluation will be done in six randomly determined fields along the implant-tissue interface.

Results obtained from in vivo test for biocompatibility evaluation of films prepared from 1% solution of oligochitosan and NOCMC are shown in Table 5 and 6 respectively.

Implants	Day-4	Day-7	Day-14	Day-21	Day-28
Inflammation	Chronic moderate	Moderate	Moderate	Few	Few
Angiogenesis	Intense	Few	Few	-	-
Granuloma	Few	-	-	-	-
Epithelioid cell	Few	Few	-	-	-
Giant cell	-	-	-	Few	Few
Fibroblast	Few	Few	Moderate	Few	Few
Implants	Intact	Mild degradation	Moderate degradation	Moderate degradation	Moderate degradation

Table 5. Oligochitosan Film: Histological Findings at Days 4,7,14,21 and 28 – Post Implantation

Implants	Day-4	Day-7	Day-14	Day-21	Day-28
Inflammation	Chronic few	Chronic few	Moderate	Moderate	Moderate
Angiogenesis	-	Few	Few	Moderate	Few
Granuloma	-	-	Few	Few	Moderate
Epithelioid cell	-	-	-	Few	Few
Giant cell	-	-	-	Few	Few
Fibroblast	Few	Few	Few	Moderate	Moderate
Implants	Intact	Mild degradation	Mild degradation	Moderate degradation	Moderate degradation

Table 6. NOCMC film- Histological Findings at Days 4,7,14,21 and 28 – Post Implantation

The inflammatory reactions seen in all the implants were fairly organized, from severe inflammation to a mild phase in the final stage of implantation. Occasionally, granuloma formation was seen in all the implants especially in the early period of implantation. Multinucleated giant cells were also present in all the implants especially towards the end of the implantation period. Angiogenesis was rather intense during the first three weeks of all implants. However, this process markedly decreased towards the end of inflammatory phase. Fibroblasts increased with longer implantation period for all of the implants and gradually decreased towards the end. However, collagen depositions were abundant especially 28 days post implantation. The implants of films prepared from oligochitosan and NOCMC undergone from mild to moderate degradation. The material was still abundantly present 28 day-post implantation. In conclusion, all film implants were biocompatible when implanted in vivo.

4.2 *In Vivo* evaluation of oligochitosan film as wound dressing

New Zealand white rabbits (n=18) with weight between 2.0 – 3.5 kg were used in this study. Four partial thickness wounds (0.5 mm, 5 x 2.5 cm2) were created using a humby knife at the lumbar region. Oligochitosan and NOCMC films were used as test material for dressing the wound and Aquacel® was used as control. The wounds were examined at day 5, day 14, and day 21.

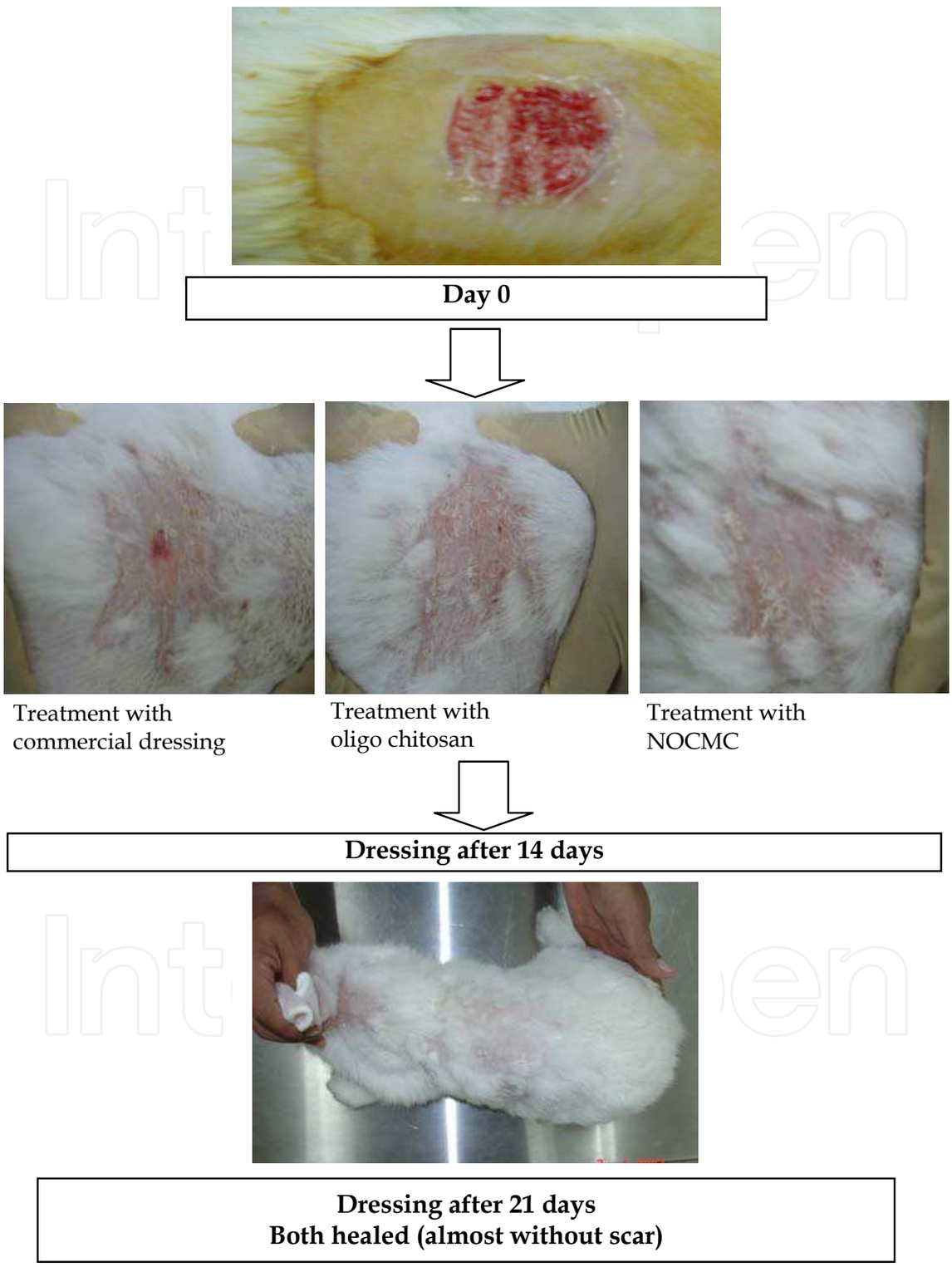


Fig. 11. Result of in vivo evaluation of NOCMC and oligochitosan films as wound dressing materials.

Split skin graft donor site is a good example of partial-thickness wound. The performances of all the chitosan derivatives as wound dressings were compared to a commercial dressing, Aquacel® which is widely used in actual clinical setting. On day 7 and 14 post-implantation with oligochitosan and NOCMC, histological findings revealed that severe neutrophilic infiltration occurred in all chitosan derivatives specimens at similar level of intensity compared with that in the control. Migration of macrophages was also accelerated by the chitosan derivatives implants. These findings were in accordance with the histological results in partial-thickness wound model where intense acute inflammations were observed in the early phase of wound healing of all the chitosan derivatives including the commercial dressing, Aquacel®. However, the inflammation intensity was significantly lower for oligochitosan compared to the treatments with NOCMC. In wound healing model, however, though fibroblasts presence in oligochitosan was lower than NOCMC, the collagen depositions were notably higher for oligochitosan which was comparable to Aquacel®.
The study on animal model for wound healing showed that both materials, oligochitosan and NOCMC are biocompatible and proved to be an effective mean of managing wound.

5. Conclusion

Chitosan is a multifunctional biopolymer with many interesting applications. However, its appeal is limited by its insolubility in neutral pH solutions and other relevant solvents. In an age where environmental considerations is of high importance, having a water soluble chitosan polymer is of great significance as it eliminates the need for employing and managing acidic solutions. The two methods employed to enhance its water solubility and processability are derivatization with identified functional groups and decreasing its chain length. Derivatization of the chitosan polymer and hydrolysis to a lower molecular weight polymer also imparts on the resulting polymer enhance activity in certain applications.
In this work we have reported on a derivatization process to produce N,O-carboxymethylchitosan and an enzymatic hydrolysis process to obtain low molecular weight chitosan polymer which we have called oligochitosan.
We have also reported on our work to establish the biological activities of the water soluble chitosan derivatives to meet two applications namely

- the ability of the derivative polymers to inhibit melanogenesis and formation of melanin
- the ability of the polymers to enhance or increase wound healing activities.

A high activity or a strong ability of the polymers to inhibit melanogenesis implies that the the polymers may be used in the development of topical products for the treatment of excess production of melanin. This occurs as melasma or simply as a darkening of the skin on exposure to environmental stress such as ultraviolet radiation or free radicals. It is possible to use the oligochitosan in the treatment of post inflammatory hyperpigmentation condition such as scar and skin darkening. In this work we have reported that the oligochitosan demonstrated a high activity in the inhibition of then formation of melanin in in-vitro cell based assays.
Exudates from non-healing wounds contain elevated levels of proteolytic enzymes, like elastase from polymorphonuclear granulocytes (PMN elastase), reactive oxygen species (ROS) and reactive nitrogen species (RNS). Thus, the reduction of protein-degrading

enzymes and scavenging of ROS and RNS seem to be suitable ways to support the healing process of chronic stagnating wounds. The water soluble polymers described in this work possess free radical scavenging activity which may assist in the treatment of chronic wound in conjunction with growth factors and hormones.
A strong ability to increase wound healing activities indicate that the polymers possess high potential to be developed as wound management products thereby increasing the choices of the medical practitioners in the treatment of chronic wounds.

6. Acknowledgements

The authors would like to acknowledge the Ministry of Science, Technology and Innovation, Malaysia for providing the grant to undertake the research described in this work. The authors would also like to express their gratitude to their respective organizations for the support given.

7. References

Boonlertniruni, S., Boobraung, C. & Suvanasara,R. (2008). Application of Chitosan in Rice Production.*Journal of Metals, Materials and Minerals,* 18, 47-52.

Brugnerotto, J., Lizardi, J., Goycoobea, F.M., Arqüelles-Monal, W., Desbrieres, J. and Rinauso, M. (2001). An infrared investigation in relation with chitin and chitosan characterization. *Polymer*, 42, 3569-3580.*Carbohydr Polym*. 66, 168-175.

Cravotto, G., Tagliapietra, S., Robaldo, B. & Trotta, M. (2005).Chemical modification of chitosan under high-intensity ultrasound. *Ultrason. Sonochem*. 12, 95-98.

Crini, G. (2005). Recent developments in polysaccharide-based materials used as adsorbents in wastewater treatment. *Prog. Polym. Sci.* 30, 38-70.

De Lima R., Feitosa,L., Santo Pereira, AE., De Moura, MR., Fauze Ahmad Aouada,FA., mattoso,LHC., Fraceto, LF.(2010). Evaluation of the Genotoxicity of Chitosan Nanoparticles for Use in Food Packaging Films. J. *Food Sci.* 75, 89-96.

El Hadrami, A., Adam, LR., El Hadrami,I., & Daayf, F. (2010). Chitosan in Plant Protection. *Mar Drugs.* 8, 968–987.

Feng, T., Du, Y., Li, J., & Kennedy, JF.(2008). Enhancement of antioxidant activity of chitosan by irradiation. *Carbohydr Polym.* 73, 126-132.

Galo Cardenas and S. Patricia Miranda (2004). FTIR and TGA studies of chitosan composite films. . *J. Chil. Chem. Soc.*, 49, 291-295.

Guo, Z., Xing, R., Liu, S., Yu, H., Wang, P., Li, C., &Li, P. (2005). The synthesis and antioxidant activity of the Schiff bases of chitosan and carboxymethyl chitosan. *Bioorg Med. Chem. Lett.* 15, 4600-4603.

Ham-Pichavant, F., Sebe, G., Pardon, P., & Coma, V.(2005). Fat resistance properties of chitosan-based paper packaging for food applications. *Carbohydr Polym*. 61, 259-265.

Hearing, V.J. (2005). Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J Dermatological Sciences,* 31, 3-14.

Hirano, S. (1999). Chitin and chitosan as novel biotechnological materials. *Polym.Int*., 48, 732-734.

Holme, H.K., Foros, H., Pettersen, H., Dornish, M., Smidsrod, O. (2001) Thermal depolymerization of chitosan chloride. Carbohydrate polym., 46, 3, 287-294.

Hsu, SC., Don, TM., & Chiu, WY. (2002). Free radical degradation of chitosan with potassium persulfate. *Polym Degrad. Stab,* .75, 73-83.

Jia, Z. & Shen, D.(2002). Effect of reaction temperature and reaction time on the preparation of low-molecular-weight chitosan using phosphoric acid.*Carbohydr. Polym*. 49, 393-396.

Kato Y., Onishi H., & Machida, Y.(2003). Application of chitin and chitosan derivatives in the pharmaceutical field. *Current Pharmaceutical Biotechnology*, 4, 303-309.

Khnor, E. & Lim L. (2003). Implantated applications of chitin and chitosan. *Biomaterials*, 24, 2339-2349.

Kittur FS, Harish Prashanth KV, Udaya Sankar K, Tharanathan RN. Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. *Carbohydrate Polymers.* 2002;49:185-193.

Kumar, M.N.V.R., Muzzarelli,R.A.A., Muzzareli,C., Sashiwa,H.,& Domb,A.J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, 104, 6017–6084.

Kwaeon, DK., Song SB., & Park YY.(2003). Preparation of water-soluble chitosan/heparin complex and its application as wound healing accelerator. *Biomaterials*, 24, 1595-1601.

Lee, Y.M., Kim, S.S., Park, M.H., Song, K.W., Sung, Y.K., Kang, I.K. β-Chitin-based wound dressing containing silver Sulfurdiazine. J Materials Science 2000;11:817-823.

Li, Q., Lunn, ET., Grandmason, EW. & Goosen MF (1997). In Applications and properties of chitosan, Goosen, MFA., Ed., Technomic Publishing Co., Inc. Lancaster, p3.

Lim, C.K., Halim, A.S., Lau, H.Y., Ujang, Z. and Hazri,A. (2007). *In vitro* cytotoxicity model of oligochitosan and N,O-carboxymethyl chitosan using primary normal human epidermal keratinocytes cultures. *Journal of Applied Biomaterials and Biomechanics*, 5, 82-87.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z. and Yao, K. D. (2001). Antibacterial action of chitosan and carboxymethylated chitosan. *Journal of Applied Polymer Science*, 79, 1324-1335.

Lopatin S. A., Ilyin M. M., Pustobaev V. N., Bezchetnikova Z. A., Varlamov V. P. and Davankov V. A. (1995). Mass-Spectrometric Analysis of *N*-Acetylchitooligosaccharides Prepared through Enzymatic Hydrolysis of Chitosan, *Anal.Biochem*., 227, 2, 285-288.

Mi,F.L., Shyu, S.S., Wu, Y.B., Lee, S.T., Shyong, J.Y. & Huang R.N.(2001) Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials*; 22:165-173.

Molinaro, G., Leroux J., Damas, J. & Adam, A.(2002). Biocompatibility of thermosensitive chitosan-based hydrogels: an in vivo experimental approach to injectable biomaterials. *Biomaterials*, 23, 2717-2722.

Mourya V.K., Inamdar, N.N and Tiwari, A. (2010). Carboxymethyl chitosan and its applications. *Advanced Materials Letter*. 1(1), 11-33.

Ng, L.T. & Swami, S. (2005). IPNs based on chitosan with NVP and NVP/HEMA synthesised through photoinitiator-free photopolymerisation technique for biomedical applications. *Carbohydr. Polym*. 60, 523-528.

Nishimura, S., Kohgo, O., Kurita, K. & Kuzuhara, H. (1991). Chemospecific manipulations of a rigid polysaccharide: syntheses of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications. *Macromolecules*, 24 , 4745–4748.

Northcott, K.A., Snape, I., Scales, P.J., & Stevens, G.W. (2005). Dewatering behaviour of water treatment sludges associated with contaminated site remediation in Antarctica.

Paulino, A.T. et. al. (2006). Characterisation of chitosan and chitin produces from silkworm chrysalides. *Carbohydr Polym*., 64. 98-103

Qin, C., Du, Y., Zong, L., Zeng, F., Lui, Y., & Zhou, B.(2003). Effect of hemicellulase on the molecular weight and structure of chitosan. *Polym Degrad. Stab.,* 80, 435-441.

Qin, C.Q., Du, Y.M., & Xiao, L. (2002). Effect of hydrogen peroxide treatment on the molecular weight and structure of chitosan. *Polym Degrad. Stab.,* 76, 211-218.

Rinoudo, M. (2006). Chitin and chitosan: Properties and applications. *Prog. Polym. Sci*. 31,603-632.

Seung, S.S., Young, C.L. and Chan, L., (2001). The degradation of chitosan with the aid of lipase from *Rhizopus japonicus* for the production of soluble chitosan. *Journal of Food Biochemistry*. 25, 307-321.

Shee, L.F.T.., Arul, J, S. Brunet and Bazinet, (2008). L. Effect of bipolar membrane electrobasification on chitosanase activity during chitosan hydrolysis, *J. of Biotech*, 134, 3-4, 305-311.

Shin- Ya, Y., Lee, M.Y., Hinode, H., & Izume, M.(2001). Effects of *N*-acetylation degree on *N*-acetylated chitosan hydrolysis with commercially available and modified pectinases. *Biochem Engineer J.*, 7, 85-88.

Suh, J.K.F. & Matthew H.W.T.(2000). Application of chitosan-based polysaccharide biomaterilas in cartilage tissue engineering: a review. *Biomaterials*, 21, 2589-2598.

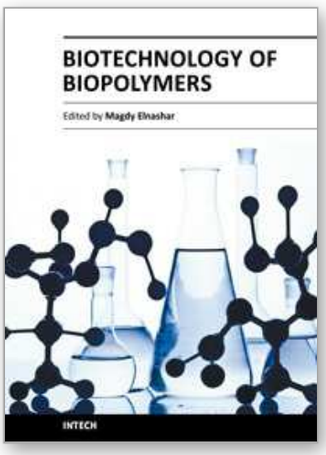
Sun, L., Du, Y., Yang, J.H., Shi, X.W., Li, J., & Wang, X.H & Kennedy, J.F. (2006). Conversion of crystal structure of the chitin to facilitate preparation of a 6-carboxychitin with moisture absorption–retention abilities. Tucci MG and Rocotti G.(2003).Chitosan and gelatin as engineered dressing for wound repair. *J. Bioactive & Compatible Polym,* 16, 145-157.

Sun T., Zhou, D., Mao, F.& Zu, Y. (2007). Preparation of low molecular weight carboxymethyl chitosan and their superoxide anion scavenging activity. *Eur. Polym. J.*, 3, 652-656.

Xie, Y., Hu, J., Wei, Y., Hong, X. (2009), Preparation of chitooligosaccharides by the enzymatic hydrolysis of chitosan, *Polymer degradation and stability*, 94,10, 1895-1899.

Zhao, Z., Wang, Z., Ye, N. and Wang, S. (2002). A novel N,O-carboxymethylchitosan amphoteric chitosan/poly(ethersulfone) composite MF membrane and its charged characteristic. *Desalinisation*, 144, 35-39.

Zhao, L., Mitomo, H., Zhai, M., Yoshii, F., Nagasawa, N., & Kume, T. (2003). Synthesis of antibacterial PVA/CM-chitosan blend hydrogels with electron beam irradiation. *Carbohydr Polym*. 53, 439-446.



Biotechnology of Biopolymers
Edited by Prof. Magdy Elnashar

ISBN 978-953-307-179-4
Hard cover, 364 pages
Publisher InTech
Published online 24, June, 2011
Published in print edition June, 2011

The book "Biotechnology of Biopolymers" comprises 17 chapters covering occurrence, synthesis, isolation and production, properties and applications, biodegradation and modification, the relevant analysis methods to reveal the structures and properties of biopolymers and a special section on the theoretical, experimental and mathematical models of biopolymers. This book will hopefully be supportive to many scientists, physicians, pharmaceutics, engineers and other experts in a wide variety of different disciplines, in academia and in industry. It may not only support research and development but may be also suitable for teaching. Publishing of this book was achieved by choosing authors of the individual chapters for their recognized expertise and for their excellent contributions to the various fields of research.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen